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(Only for new nonprovisional applications under 37 CFR 1.53(b))

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First Named Inventor Gillies

Title Enhancing the Circulating Half Life of Antibody-
Based Fusion Proteins

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APPLICATION ELEMENTS

ADDRESS TO: **Box Patent Application**
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Washington, D.C. 20231

1. ☐ Fee Transmittal Form

2. ☒ Specification and Drawings [Total Pages 38]
- Specification - (18 pages)
- Claims - (4 pages)
- Abstract - (1 page)
- Sheets of Drawings - (7 sheets)
☐ Formal
☒ Informal
- Sequence Listing - (8 sheets)

3. ☐ Oath or Declaration [Total Pages]
a. ☐ Newly executed (original)
b. ☐ Copy from a prior application (37 CFR 1.63(d))
(for continuation/divisional with Box 17 completed)
[Note Box 4 below]

4. ☐ Incorporation by Reference (usable if Box 3b is checked)
The entire Disclosure of the prior application, from
which a copy of the oath or declaration is supplied
under Box 3b, is considered as being part of the
disclosure of the accompanying application and is
hereby incorporated by reference therein.

5. ☐ Microfiche Computer Program (Appendix)

6. ☒ Nucleotide and/or Amino Acid Sequence Submission
☒ Computer Readable Copy
☒ Paper Copy (identical to computer copy)
☒ Statement verifying identity of above copies

ACCOMPANYING APPLICATION PARTS

7. ☐ 37 CFR 3.73(b) Statement (when there is an assignee)
☐ Power of Attorney

8. ☐ English Translation Document (if applicable)

9. ☐ Information Disclosure Statement (IDS)/PTO-1449
☐ Copies of IDS Citations

10. ☐ Preliminary Amendment
☐ Drawings [Total Sheets]
☐ Letter to Official Draftsperson Including
Drawings [Total Pages]

11. ☒ Return Receipt Postcard

12. ☐ Small Entity Statement(s)
☐ Statements filed in prior application,
(Status still proper and desired)

13. ☐ Certified Copy of Priority Document(s)

14. ☐ Deletion of Inventor(s)
Signed statement attached deleting inventor(s) named in
the prior application.

15. ☒ Patent Application Data Entry Form (2 sheets)

16. ☐ Other:

17. ☐ If a **CONTINUING APPLICATION**, check appropriate box and supply the requisite information:

☐ Continuation ☐ Divisional ☐ Continuation-in-part (CIP) of prior application Serial No. ____/____.

Priority to the above application(s) is claimed under 35 U.S.C. 120.

Prior application information: Examiner: _____. Group/Art Unit: _____.

18. ☒ **Priority - 35 U.S.C. 119**

☒ Priority of application U.S. Serial No. 60/075,887 filed on February 25, 1998 is claimed under 35 U.S.C. 119.

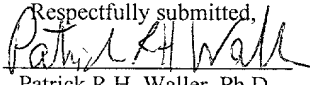
☐ The certified copy has been filed in prior U.S. application Serial No. ____/____ on ____.

☐ The certified copy will follow.

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This application claims priority to::
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ENHANCING THE CIRCULATING HALF-LIFE OF ANTIBODY-BASED FUSION PROTEINS

Cross Reference to Related Application

This incorporates by reference, and claims priority to and the benefit of, U.S. Provisional Patent Application Serial Number 60/075,887 which was filed on February 25, 1998.

Field of the Invention

The present invention relates generally to fusion proteins. More specifically, the present invention relates to methods of enhancing the circulating half-life of antibody-based fusion proteins.

Background of the Invention

The use of antibodies for treatment human disease is well established and has become more sophisticated with the introduction of genetic engineering. Several techniques have been developed to improve the utility of antibodies. These include: (1) the generation of monoclonal antibodies by cell fusion to create "hybridomas", or by molecular cloning of antibody heavy (H) and light (L) chains from antibody-producing cells; (2) the conjugation of other molecules to antibodies to deliver them to preferred sites *in vivo*, e.g., radioisotopes, toxic drugs, protein toxins, and cytokines; (3) the manipulation of antibody effector functions to enhance or diminish biological activity; (4) the joining of other protein such as toxins and cytokines with antibodies at the genetic level to produce antibody-based fusion proteins; and (5) the joining of one or more sets of antibody combining regions at the genetic level to produce bi-specific antibodies.

When proteins are joined together through either chemical or genetic manipulation, it is often difficult to predict what properties that the end product will

retain from the parent molecules. With chemical conjugation, the joining process may occur at different sites on the molecules, and generally results in molecules with varying degrees of modification that can affect the function of one or both proteins. The use of genetic fusions, on the other hand, makes the joining process more consistent, and results in the production of consistent end products that retain the function of both component proteins. *See*, for example, Gillies *et al.*, PROC. NATL. ACAD. SCI. USA 89: 1428-1432 (1992); and U.S. Patent No. 5,650,150.

However, the utility of recombinantly-produced antibody-based fusion proteins may be limited by their rapid *in vivo* clearance from the circulation. Antibody-cytokine fusion proteins, for example, have been shown to have a significantly lower *in vivo* circulating half-life than the free antibody. When testing a variety of antibody-cytokine fusion proteins, Gillies *et al.* reported that all of the fusion proteins tested had an α phase (distribution phase) half-life of less than 1.5 hour. Indeed, most of the antibody-based fusion protein were cleared to 10% of the serum concentration of the free antibody by two hours. *See*, Gillies *et al.*, BIOCONJ. CHEM. 4: 230-235 (1993). Therefore, there is a need in the art for methods of enhancing the *in vivo* circulating half-life of antibody-based fusion proteins.

Summary of the Invention

A novel approach to enhancing the *in vivo* circulating half-life of antibody-based fusion proteins has now been discovered. Specifically, the present invention provides methods for the production of fusion proteins between an immunoglobulin with a reduced binding affinity for an Fc receptor, and a second non-immunoglobulin protein. Antibody-based fusion proteins with reduced binding affinity for Fc receptors have a significantly longer *in vivo* circulating half-life than the unlinked second non-immunoglobulin protein.

IgG molecules interact with three classes of Fc receptors (FcR) specific for the IgG class of antibody, namely Fc γ RI, Fc γ RII and Fc γ RIII. In preferred embodiments, the immunoglobulin (Ig) component of the fusion protein has at least a portion of the

constant region of an IgG that has a reduced binding affinity for at least one of FcγRI, FcγRII or FcγRIII.

In one aspect of the invention, the binding affinity of fusion proteins for Fc receptors is reduced by using heavy chain isotypes as fusion partners that have reduced binding affinity for Fc receptors on cells. For example, both human IgG1 and IgG3 have been reported to bind to FcRγI with high affinity, while IgG4 binds 10-fold less well, and IgG2 does not bind at all. The important sequences for the binding of IgG to the Fc receptors have been reported to be located in the CH2 domain. Thus, in a preferred embodiment, an antibody-based fusion protein with enhanced *in vivo* circulating half-life is obtained by linking at least the CH2 domain of IgG2 or IgG4 to a second non-immunoglobulin protein.

In another aspect of the invention, the binding affinity of fusion proteins for Fc receptors is reduced by introducing a genetic modification of one or more amino acid in the constant region of the IgG1 or IgG3 heavy chains that reduces the binding affinity of these isotypes for Fc receptors. Such modifications include alterations of residues necessary for contacting Fc receptors or altering others that affect the contacts between other heavy chain residues and Fc receptors through induced conformational changes. Thus, in a preferred embodiment, an antibody-based fusion protein with enhanced *in vivo* circulating half-life is obtained by first introducing a mutation, deletion, or insertion in the IgG1 constant region at one or more amino acid selected from Leu₂₃₄, Leu₂₃₅, Gly₂₃₆, Gly₂₃₇, Asn₂₉₇, and Pro₃₃₁, and then linking the resulting immunoglobulin, or portion thereof, to a second non-immunoglobulin protein. In an alternative preferred embodiment, the mutation, deletion, or insertion is introduced in the IgG3 constant region at one or more amino acid selected from Leu₂₈₁, Leu₂₈₂, Gly₂₈₃, Gly₂₈₄, Asn₃₄₄, and Pro₃₇₈, and the resulting immunoglobulin, or portion thereof, is linked to a second non-immunoglobulin protein. The resulting antibody-based fusion proteins have a longer *in vivo* circulating half-life than the unlinked second non-immunoglobulin protein.

In a preferred embodiment, the second non-immunoglobulin component of the fusion protein is a cytokine. The term “cytokine” is used herein to describe proteins,

analogs thereof, and fragments thereof which are produced and excreted by a cell, and which elicit a specific response in a cell which has a receptor for that cytokine.

Preferably, cytokines include interleukins such as interleukin-2 (IL-2), hematopoietic factors such as granulocyte-macrophage colony stimulating factor (GM-CSF), tumor
5 necrosis factor (TNF) such as $\text{TNF}\alpha$, and lymphokines such as lymphotoxin. Preferably, the antibody-cytokine fusion protein of the present invention displays cytokine biological activity.

In an alternative preferred embodiment, the second non-immunoglobulin component of the fusion protein is a ligand-binding protein with biological activity. Such
10 ligand-binding proteins may, for example, (1) block receptor-ligand interactions at the cell surface; or (2) neutralize the biological activity of a molecule (*e.g.*, a cytokine) in the fluid phase of the blood, thereby preventing it from reaching its cellular target.

Preferably, ligand-binding proteins include CD4, CTLA-4, TNF receptors, or interleukin receptors such as the IL-1 and IL-4 receptors. Preferably, the antibody-receptor fusion
15 protein of the present invention displays the biological activity of the ligand-binding protein.

In yet another alternative preferred embodiment, the second non-immunoglobulin component of the fusion protein is a protein toxin. Preferably, the antibody-toxin fusion protein of the present invention displays the toxicity activity of the protein toxin.

20 In a preferred embodiment, the antibody-based fusion protein comprises a variable region specific for a target antigen and a constant region linked through a peptide bond to a second non-immunoglobulin protein. The constant region may be the constant region normally associated with the variable region, or a different one, *e.g.*, variable and constant regions from different species. The heavy chain can include a CH1, CH2, and/or
25 CH3 domains. Also embraced within the term "fusion protein" are constructs having a binding domain comprising framework regions and variable regions (*i.e.*, complementarity determining regions) from different species, such as are disclosed by Winter, *et al.*, GB 2,188, 638. Antibody-based fusion proteins comprising a variable region preferably display antigen-binding specificity. In yet another preferred

embodiment, the antibody-based fusion protein further comprises a light chain. The invention thus provides fusion proteins in which the antigen-binding specificity and activity of an antibody are combined with the potent biological activity of a second non-immunoglobulin protein, such as a cytokine. A fusion protein of the present invention
5 can be used to deliver selectively the second non-immunoglobulin protein to a target cell *in vivo* so that the second non-immunoglobulin protein can exert a localized biological effect.

In an alternative preferred embodiment, the antibody-based fusion protein comprises a heavy chain constant region linked through a peptide bond to a second non-immunoglobulin protein, but does not comprise a heavy chain variable region. The
10 invention thus further provides fusion proteins which retain the potent biological activity of a second non-immunoglobulin protein, but which lack the antigen-binding specificity and activity of an antibody.

In preferred embodiments, the antibody-based fusion proteins of the present
15 invention further comprise sequences necessary for binding to Fc protection receptors (FcRp), such as beta-2 microglobulin-containing neonatal intestinal transport receptor (FcRn).

In preferred embodiments, the fusion protein comprises two chimeric chains comprising at least a portion of a heavy chain and a second, non-Ig protein are linked by a
20 disulfide bond.

The invention also features DNA constructs encoding the above-described fusion proteins, and cell lines, *e.g.*, myelomas, transfected with these constructs.

These and other objects, along with advantages and features of the invention disclosed herein, will be made more apparent from the description, drawings, and claims
25 that follow.

Brief Description of the Drawings

The foregoing and other objects, features, and advantages of the present invention, as well as the invention itself, may be more fully understood from the following description of preferred embodiments, when read together with the accompanying drawings, in which:

FIG. 1 is a homology alignment of the amino acid sequences of the constant region of C γ 1 and C γ 3, aligned to maximize amino acid identity, and wherein non-conserved amino acids are identified by boxes;

FIG. 2 is a homology alignment of the amino acid sequences of constant region of C γ 1, C γ 2, and C γ 4, aligned to maximize amino acid identity, and wherein non-conserved amino acids are identified by boxes;

FIG. 3 is a diagrammatic representation of a map of the genetic construct encoding an antibody-based fusion protein showing the relevant restriction sites;

FIG. 4 is a bar graph depicting the binding of antibody hu-KS-1/4 and antibody-based fusion proteins, hu-KS γ 1-IL2 and hu-KS γ 4-IL2, to Fc receptors on mouse J774 cells in the presence (solid bars) or absence (stippled bars) of an excess of mouse IgG;

FIG. 5 is a line graph depicting the *in vivo* plasma concentration of total antibody (free antibody and fusion protein) of hu-KS γ 1-IL2 (closed diamond) and hu-KS γ 4-IL2 (closed triangle) and of intact fusion protein of hu-KS γ 1-IL2 (open diamond) and hu-KS γ 4-IL2 (open triangle) as a function of time;

FIG. 6 is a diagrammatic representation of protocol for constructing an antibody-based fusion protein with a mutation that reduces the binding affinity to Fc receptors;

FIG. 7 is a line graph depicting the *in vivo* plasma concentration of intact fusion protein of hu-KS γ 1-IL2 (\diamond); mutated hu-KS γ 1-IL2 (\square) and hu-KS γ 4-IL2 (Δ) as a function of time.

Detailed Description of the Invention

It has now been discovered that fusing a second protein, such as a cytokine, to an immunoglobulin may alter the antibody structure, resulting in an increase in binding affinity for one or more of the cell-bound Fc receptors and leading to a rapid clearance of the antibody-based fusion protein from the circulation. The present invention describes antibody-based fusion proteins with enhanced *in vivo* circulating half-lives and involves producing, through recombinant DNA technology, antibody-based fusion proteins with reduced binding affinity for one or more Fc receptor.

First, an antibody-based fusion protein with an enhanced *in vivo* circulating half-life can be obtained by constructing a fusion protein with isotypes having reduced binding affinity for a Fc receptor, and avoiding the use of sequences from antibody isotypes that bind to Fc receptors. For example, of the four known IgG isotypes, IgG1 (C γ 1) and IgG3 (C γ 3) are known to bind FcR γ I with high affinity, whereas IgG4 (C γ 4) has a 10-fold lower binding affinity, and IgG2 (C γ 2) does not bind to FcR γ I. Thus, an antibody-based fusion protein with reduced binding affinity for a Fc receptor could be obtained by constructing a fusion protein with a C γ 2 constant region (Fc region) or a C γ 4 Fc region, and avoiding constructs with a C γ 1 Fc region or a C γ 3 Fc region.

Second, an antibody-based fusion protein with an enhanced *in vivo* circulating half-life can be obtained by modifying sequences necessary for binding to Fc receptors in isotypes that have binding affinity for an Fc receptor, in order to reduce or eliminate binding. As mentioned above, IgG molecules interact with three classes of Fc receptors (FcR), namely FcR γ I, FcR γ II, and FcR γ III. C γ 1 and C γ 3 bind FcR γ I with high affinity, whereas C γ 4 and C γ 2 have reduced or no binding affinity for FcR γ I. A comparison of the C γ 1 and C γ 3 indicates that, with the exception of an extended hinge segment in C γ 3, the amino acid sequence homology between these two isotypes is very high. This is true even in those regions that have been shown to interact with the C1q fragment of complement and the various FcR classes. FIG. 1 provides a alignment of the amino acid sequences of C γ 1 and C γ 3. The other two isotypes of human IgG (C γ 2 and C γ 4) have sequence differences which have been associated with FcR binding. FIG. 2 provides a

alignment of the amino acid sequences of C γ 1, C γ 2, and C γ 4. The important sequences for Fc γ R binding are Leu-Leu-Gly-Gly (residues 234 through 237 in C γ 1), located in the CH2 domain adjacent to the hinge. Canfield and Morrison, J. EXP. MED. 173: 1483-1491 (1991). These sequence motifs are conserved in C γ 1 and C γ 3, in agreement with their similar biological properties, and possibly related to the similarity of pharmacokinetic behavior when used to construct IL-2 fusion proteins. Many mutational analyses have been done to demonstrate the effect of specific mutations on FcR binding, including those in residues 234-237 as well as the hinge-proximal bend residue Pro₃₃₁ that is substituted by Ser in IgG4. Another important structural component necessary for effective FcR binding is the presence of an N-linked carbohydrate chain covalently bound to Asn₂₉₇. Enzymatic removal of this structure or mutation of the Asn residue effectively abolish, or at least dramatically reduce, binding to all classes of Fc γ R.

Brumbell *et al.* postulated the existence of a protection receptor (FcRp) that would slow the rate of catabolism of circulating antibodies by binding to the Fc portion of antibodies and, following their pinocytosis into cells, would redirect them back into the circulation. Brumbell *et al.*, NATURE 203: 1352-1355 (1964). The beta-2 microglobulin-containing neonatal intestinal transport receptor (FcRn) has recently been identified as a FcRp. See, Junghans *et al.*, PROC. NATL. ACAD. SCI. USA 93: 5512-5516 (1996). The sequences necessary for binding to this receptor are conserved in all four classes of human IgG and are located at the interface between the CH2 and CH3 domains. See, Medesan *et al.*, J. IMMUNOL. 158: 2211-2217 (1997). These sequences have been reported to be important for the *in vivo* circulating half-life of antibodies. See, International PCT publication WO 97/34631. Thus, preferred antibody-based fusion proteins of the present invention will have the sequences necessary for binding to FcRp.

Methods for synthesizing useful embodiments of the invention are described, as well as assays useful for testing their pharmacokinetic activities, both *in vitro* and in pre-clinical *in vivo* animal models. The preferred gene construct encoding a chimeric chain includes, in 5' to 3' orientation, a DNA segment which encodes at least a portion of an immunoglobulin and DNA which encodes a second, non-immunoglobulin protein. An

alternative preferred gene construct includes, in 5' to 3' orientation, a DNA segment which encodes a second, non-immunoglobulin protein and DNA which encodes at least a portion of an immunoglobulin. The fused gene is assembled in or inserted into an expression vector for transfection of the appropriate recipient cells where it is expressed.

5 The invention is illustrated further by the following non-limiting examples:

Example 1 Improving the *in vivo* circulating half-life of an antibody-IL2 fusion protein by class switching from C γ 1 to C γ 4 IgG constant regions.

According to the present invention, antibody-based fusion proteins with enhanced *in vivo* circulating half-lives can be obtained by constructing antibody-based fusion
10 proteins using sequences from antibody isotypes that have reduced or no binding affinity for Fc receptors.

In order to assess whether the *in vivo* circulating half-life of the antibody-based fusion protein can be enhanced by using sequences from antibody isotypes with reduced or no binding affinity for Fc receptors, an antibody-IL2 fusion protein with a human C γ 1 constant region (Fc region) was compared to an antibody-IL2 fusion protein with a
15 human C γ 4 Fc region.

1.1 Construction of antibody-IL2 fusion proteins with a C γ 4 IgG constant region.

The construction of antibody-IL2 fusion proteins with a C γ 1 constant region has
20 been described in the prior art. *See*, for example, Gillies *et al.*, PROC. NATL. ACAD. SCI. USA 89: 1428-1432 (1992); and U.S. Patent No 5,650,150, the disclosure of which is incorporated herein by reference.

To construct antibody-IL2 fusion proteins with a C γ 4 constant region, a plasmid vector, capable of expressing a humanized antibody-IL2 fusion protein with variable (V)
25 regions specific for a human pancreatic carcinoma antigen (KSA) and the human C γ 1 heavy chain fused to human IL-2, was modified by removing the C γ 1 gene fragment and replacing it with the corresponding sequence from the human C γ 4 gene. A map of some of the relevant restriction sites and the site of insertion of the C γ 4 gene fragment is

provided in FIG. 3. These plasmid constructs contain the cytomegalovirus (CMV) early promoter for transcription of the mRNA encoding the light (L) and heavy (H) chain variable (V) regions derived from the mouse antibody KS-1/4. The mouse V regions were humanized by standard methods and their encoding DNA sequences were

5 chemically synthesized. A functional splice donor site was added at the end of each V region so that it could be used in vectors containing H and L chain constant region genes. The human C κ light chain gene was inserted downstream of the cloning site for the VL gene and was followed by its endogenous 3' untranslated region and poly adenylation site. This transcription unit was followed by a second independent transcription unit for

10 the heavy chain-IL2 fusion protein. It is also driven by a CMV promoter. The VH encoding sequence was inserted upstream of the DNA encoding the C γ heavy chain gene of choice, fused to human IL-2 encoding sequences. Such C γ genes contain splice acceptor sites for the first heavy chain exon (CH1), just downstream from a unique Hind III common to all human C γ genes. A 3' untranslated and polyadenylation site from

15 SV40 virus was inserted at the end of the IL-2 encoding sequence. The remainder of the vector contained bacterial plasmid DNA necessary for propagation in *E. coli* and a selectable marker gene (dihydrofolate reductase - dhfr) for selection of transfectants of mammalian cells.

The swapping of the C γ 1 and C γ 4 fragments was accomplished by digesting the

20 original C γ 1-containing plasmid DNA with Hind III and Xho I and purifying the large 7.8 kb fragment by agarose gel electrophoresis. A second plasmid DNA containing the C γ 4 gene was digested with Hind III and Nsi I and the 1.75 kb fragment was purified. A third plasmid containing the human IL-2 cDNA and SV40 poly A site, fused to the carboxyl terminus of the human C γ 1 gene, was digested with Xho I and Nsi I and the

25 small 470 bp fragment was purified. All three fragments were ligated together in roughly equal molar amounts and the ligation product was used to transform competent *E. coli*. The ligation product was used to transform competent *E. coli* and colonies were selected by growth on plates containing ampicillin. Correctly assembled recombinant plasmids were identified by restriction analyses of plasmid DNA preparations from isolated

transformants and digestion with Fsp I was used to discriminate between the C γ 1 (no Fsp I) and C γ 4 (one site) gene inserts. The final vector, containing the C γ 4-IL2 heavy chain replacement, was introduced into mouse myeloma cells and transfectants were selected by growth in medium containing methotrexate (0.1 μ M). Cell clones expressing high levels of the antibody-IL2 fusion protein were expanded and the fusion protein was purified from culture supernatants using protein A Sepharose chromatography. The purity and integrity of the C γ 4 fusion protein was determined by SDS-polyacrylamide gel electrophoresis. IL-2 activity was measured in a T-cell proliferation assay and found to be identical to that of the C γ 1 construct.

1.2 Binding to Fc receptors by antibody and antibody-IL2 fusion proteins with C γ 1 and C γ 4 IgG constant region.

Various mouse and human cell lines express one or more Fc receptor. For example, the mouse J774 macrophage-like cell line expresses FcR γ I that is capable of binding mouse or human IgG of the appropriate subclasses. Likewise, the human K562 erythroleukemic cell line expresses FcR γ II but not FcR γ I. In order to assess the potential contribution of Fc receptor binding to clearance of antibody-based fusion proteins from the circulation, the binding affinities of an antibody, a C γ 1-IL2 fusion protein, and a C γ 4-IL2 fusion protein for FcR γ I were compared in the mouse J774 cell line.

The two antibody-IL-2 fusion proteins described in Example 1, hu-KS γ 1-IL2 and hu-KS γ 4-IL2, were diluted to 2 μ g/ml in PBS containing 0.1% bovine serum albumin (BSA), together with 2×10^5 J774 cells in a final volume of 0.2 ml. After incubation on ice for 20 min, a FITC-conjugated anti-human IgG Fc antibody (Fab₂) was added and incubation was continued for an additional 30 min. Unbound antibodies were removed by two washes with PBS-BSA, and the cells were analyzed in a fluorescence-activated cell sorter (FACS). Control reactions contained the same cells mixed with just the FITC-labeled secondary antibody or with the humanized KS γ 1 antibody (without IL-2).

As expected, the binding of the C γ 4-IL2 fusion protein to J774 cells was significantly lower than the binding of the C γ 1-IL2 fusion protein. See FIG. 4. Unexpectedly, however, both the C γ 1-IL2 and C γ 4-IL2 fusion proteins had significantly

higher binding to J774 cells than the K Σ 1 antibody (without IL-2). This suggests that fusing a second protein, such as a cytokine, to an immunoglobulin may alter the antibody structure, resulting in an increase in binding affinity for one or more of the cell-bound Fc receptors, thereby leading to a rapid clearance from the circulation.

5 In order to determine whether the greater binding observed with IL-2 fusion proteins was due to the presence of IL-2 receptors or FcR γ 1 receptors on the cells, excess mouse IgG (mIgG) was used to compete the binding at the Fc receptors. As illustrated in FIG. 4, background levels of binding were observed with the antibody and both antibody-IL2 fusion proteins in the presence of a 50-fold molar excess of mIgG. This
10 suggests that the increased signal binding of antibody-IL2 fusion proteins was due to increased binding to the Fc receptor.

Cell lines expressing Fc receptors are useful for testing the binding affinities of candidate fusion proteins to Fc receptors in order to identify antibody-based fusion proteins with enhanced *in vivo* half lives. Candidate antibody-based fusion proteins can
15 be tested by the above-described methods. Candidate antibody-based fusion proteins with substantially reduced binding affinity for an Fc receptor will be identified as antibody-based fusion proteins with enhanced *in vivo* half lives.

1.3 Measuring the circulating half-life of antibody-IL2 fusion proteins with C γ 1 and C γ 4 IgG constant region.

20 In order to assess whether using the Fc region of an IgG isotype having reduced affinity for Fc receptors will enhance the *in vivo* circulating half-life, fusion proteins containing the C γ 1 isotype heavy chain (*i.e.*, hu-K Σ 1-IL2) were compared to fusion proteins containing the C γ 4 isotype heavy chain (*i.e.*, hu-K Σ 4-IL2).

Purified humanized KS-1/4-IL2 fusion proteins containing either the C γ 1 or C γ 4
25 isotype heavy chain were buffer-exchanged by diafiltration into phosphate buffered saline (PBS) and diluted further to a concentration of ~100 μ g/ml. Approximately 20 μ g of the antibody-based fusion protein (0.2 ml) was injected into 6-8 week old Balb/c mice in the tail vein using a slow push. Four mice were injected per group. At various time points,

small blood samples were taken by retro-orbital bleeding from anaesthetized animals and collected in tubes containing citrate buffer to prevent clotting. Cells were removed by centrifugation in an Eppendorf high-speed tabletop centrifuge for 5 min. The plasma was removed with a micropipettor and frozen at -70°C . The concentration of human antibody determinants in the mouse blood was measured by ELISA. A capture antibody specific for human H and L antibody chains was used for capture of the fusion proteins from the diluted plasma samples. After a two hour incubation in antibody-coated 96-well plates, the unbound material was removed by three washes with ELISA buffer (0.01% Tween 80 in PBS). A second incubation step used either an anti-human Fc antibody (for detection of both antibody and intact fusion protein), or an anti-human IL-2 antibody (for detection of only the intact fusion protein). Both antibodies were conjugated to horse radish peroxidase (HRP). After a one hour incubation, the unbound detecting antibody was removed by washing with ELISA buffer and the amount of bound HPR was determined by incubation with substrate and measuring in a spectrophotometer.

As depicted in FIG. 5, the α phase half-life of the hu-KS γ 4-IL2 fusion protein was significantly longer than the α phase half-life of the hu-KS γ 1-IL2 fusion protein. The increased half-life is best exemplified by the significantly higher concentrations of the hu-KS γ 4-IL2 fusion protein (3.3 $\mu\text{g/ml}$) compared to the hu-KS γ 1-IL2 fusion protein (60 ng/ml) found in mice after 24 hours.

The hu-KS γ 1-IL2 protein had a rapid distribution (α) phase followed by a slower catabolic (β) phase, as reported earlier for the chimeric 14.18-IL2 fusion protein. *See*, Gillies *et al.*, BIOCONJ. CHEM. 4: 230-235 (1993). In the Gillies *et al.* study, only antibody determinants were measured, so it was not clear if the clearance represented the clearance of the intact fusion protein or the clearance of the antibody component of the fusion protein. In the present Example, samples were assayed using both (1) an antibody-specific ELISA, and (2) a fusion protein-specific ELISA (*i.e.*, an ELISA that requires that both the antibody and IL-2 components be physically linked). As illustrated in FIG. 5, in animals injected with the hu-KS γ 1-IL2 fusion protein, the amount of circulating fusion protein was lower than the total amount of circulating antibody, especially at the 24 hr

time point. This suggests that the fusion protein is being proteolytically cleaved *in vivo* and that the released antibody continues to circulate. Surprisingly, in animals injected with the hu-KS γ 4-IL2 fusion protein, there was no significant differences between the amount of circulating fusion protein and the total amount of circulating antibody. This suggests the hu-KS γ 4-IL2 fusion protein was not being proteolytically cleaved in these animals during the 24 hour period measured.

As discussed above, C γ 1 and C γ 3 have binding affinity for Fc receptors, whereas while C γ 4 has reduced binding affinity and C γ 2 has no binding affinity for Fc receptors. The present Example described methods for producing antibody-based fusion proteins using the C γ 4 Fc region, an IgG isotype having reduced affinity for Fc receptors, and established that such antibody-based fusion proteins have enhanced *in vivo* circulating half-life. Accordingly, a skilled artisan can use these methods to produce antibody-based fusion proteins with the C γ 2 Fc region, instead of the C γ 4 Fc region, in order to enhance the circulating half-life of fusion proteins. A Hu-KS-IL2 fusion protein utilizing the human C γ 2 region can be constructed using the same restriction fragment replacement and the above-described methods for C γ 4-IL2 fusion protein. and tested using the methods described herein to demonstrate increased circulating half-life. Antibody-based fusion proteins with the C γ 2 Fc region, or any other Fc region having reduced binding affinity or lacking binding affinity for a Fc receptor will have enhanced *in vivo* circulating half-life compared to antibody-based fusion proteins having binding affinity for a Fc receptor.

Example 2 Mutating the human C γ 1 or C γ 3 gene in antibody-based fusion protein constructs to improve their *in vivo* circulating half-life.

IgG molecules interact with several molecules in the circulation, including members of the complement system of proteins (*e.g.*, C1q fragment), as well as the three classes of FcR. The important residues for C1q binding are residues Glu₃₁₈, Lys₃₂₀, and Lys₃₂₂ which are located in the CH2 domains of human heavy chains. Tao *et al.*, J. EXP. MED. 178: 661-667 (1993). In order to discriminate between FcR and C1q binding as mechanisms for rapid clearance, we substituted the more drastically altered C γ 2

hinge-proximal segment into the C γ 1 heavy chain. This mutation is expected to affect FcR binding but not complement fixation.

The mutation was achieved by cloning and adapting the small region between the hinge and the beginning of the CH2 exon of the germ line C γ 1 gene using overlapping polymerase chain reactions (PCR). The PCR primers were designed to substitute the new sequence at the junction of two adjacent PCR fragments spanning a Pst I to Drd I fragment (see FIG. 6). In the first step, two separate PCR reactions with primers 1 and 2 (SEQ ID NOS: 5 and 6, respectively), or primers 3 and 4 (SEQ ID NOS: 7 and 8, respectively), were prepared using the C γ 1 gene as the template. The cycle conditions for the primary PCR were 35 cycles of: 94°C for 45 sec, annealing at 48°C for 45 seconds, and primer extension at 72°C for 45 sec. The products of each PCR reaction were used as template for the second, joining reaction step. One tenth of each primary reaction was mixed together and combined with primers 1 and 4 to amplify only the combined product of the two initial PCR products. The conditions for the secondary PCR were: 94°C for 1 min, annealing at 51°C for 1 min, and primer extension at 72°C for 1 min. Joining occurs as a result of the overlapping between the two individual fragments which pairs with the end of the other, following denaturation and annealing. The fragments that form hybrids get extended by the Taq polymerase, and the complete, mutated product was selectively amplified by the priming of the outer primers, as shown in FIG. 6. The final PCR product was cloned in a plasmid vector and its sequence verified by DNA sequence analysis.

The assembly of the mutated gene was done in multiple steps. In the first step, a cloning vector containing the human C γ 1 gene was digested with Pst I and Xho I to remove the non-mutated hinge-CH2-CH3 coding sequences. A Drd I to Xho I fragment encoding part of CH2, all of CH3 and the fused human IL-2 coding sequences was prepared from the C γ 1-IL2 vector, described above. A third fragment was prepared from the subcloned PCR product by digestion with Pst I and Drd I. All three fragments were purified by agarose gel electrophoresis and ligated together in a single reaction mixture. The ligation product was used to transform competent *E. coli* and colonies were selected

by growth on plates containing ampicillin. Correctly assembled recombinant plasmids were identified by restriction analyses of plasmid DNA preparations from isolated transformants and mutated genes were confirmed by DNA sequence analysis. The Hind III to Xho I fragment from the mutated C γ 1-IL2 gene was used to reassemble the complete hu-KS antibody-IL2 fusion protein expression vector.

In order to assess the enhancement of the *in vivo* circulating half-life induced by a mutation of an important amino acid for FcR binding, and to discriminate between FcR and C1q binding as mechanisms for rapid clearance, the *in vivo* plasma concentration of the mutated hu-KS γ 1-IL2 was compared to the plasma concentration of hu-KS γ 1-IL2 at various specified times. As illustrated in FIG. 7, the *in vivo* clearance rates of the mutated hu-KS γ 1-IL2 and hu-KS γ 4-IL2 were significantly lower than the clearance rate of hu-KS γ 1-IL2. These results suggests that an antibody-based fusion protein with enhanced *in vivo* circulating half-life can be obtained by modifying sequences necessary for binding to Fc receptors in isotypes that have binding affinity for an Fc receptor. Further, the results suggests that the mechanisms for rapid clearance involve FcR binding rather than C1q binding.

The skilled artisan will understand, from the teachings of the present invention, that several other mutations to the C γ 1 or C γ 3 genes can be introduced in order to reduce binding to FcR and enhance the *in vivo* circulating half-life of an antibody-based fusion protein. Moreover, mutations can also be introduced into the C γ 4 gene in order to further reduce the binding of C γ 4 fusion proteins to FcR. For example, additional possible mutations include mutations in the hinge proximal amino acid residues, mutating Pro₃₃₁, or by mutating the single N-linked glycosylation site in all IgG Fc regions. The latter is located at Asn₂₉₇ as part of the canonical sequence: Asn-X-Thr/Ser, where the second position can be any amino acid (with the possible exception of Pro), and the third position is either Thr or Ser. A conservative mutation to the amino acid Gln, for example, would have little effect on the protein but would prevent the attachment of any carbohydrate side chain. A strategy for mutating this residue might follow the general procedure, just described, for the hinge proximal region. Methods for generating point mutations in

cloned DNA sequences are well established in the art and commercial kits are available from several vendors for this purpose.

Example 3 Increasing the circulating half-life of receptor-antibody-based fusion proteins.

5 Several references have reported that the Fc portion of human IgG can serve as a useful carrier for many ligand-binding proteins, or receptors, with biological activity. Some of these ligand-binding proteins have been fused to the N-terminal of the Fc portion of an Ig, such as CD4, CTLA-4, and TNF receptors. *See*, for example, Capon *et al.*, NATURE 337: 525-531 (1989); Linsley *et al.*, J. EXP. MED. 174: 561-569 (1991);
10 Wooley *et al.*, J. IMMUNOL. 151: 6602-6607 (1993). Increasing the circulating half-life of receptor-antibody-based fusion proteins may permit the ligand-binding protein partner (*i.e.*, the second non-Ig protein) to more effectively (1) block receptor-ligand interactions at the cell surface; or (2) neutralize the biological activity of a molecule (*e.g.*, a cytokine) in the fluid phase of the blood, thereby preventing it from reaching its cellular target. In
15 order to assess whether reducing the ability of receptor-antibody-based fusion proteins to bind to IgG receptors will enhance their *in vivo* circulating half-life, receptor-antibody-based fusion proteins with human C γ 1 Fc regions are compared to antibody-based fusion proteins with human C γ 4 Fc regions.

To construct CD4-antibody-based fusion proteins, the ectodomain of the human
20 CD4 cell surface receptor is cloned using PCR from human peripheral blood monocyctic cells (PBMC). The cloned CD4 receptor includes compatible restriction sites and splice donor sites described in Example 1. The expression vector contains a unique Xba I cloning site downstream of the CMV early promoter, and the human C γ 1 or C γ 4 gene downstream of their endogenous Hind III site. The remainder of the plasmid contains
25 bacterial genetic information for propagation in *E. coli*, as well as a dhfr selectable marker gene. Ligated DNAs are used to transform competent bacteria and recombinant plasmids are identified from restriction analyses from individual bacterial colonies. Two plasmid DNA constructs are obtained: CD4-C γ 1 and CD4-C γ 4.

The expression plasmids are used to transfect mouse myeloma cells by electroporation and transfectants are selected by growth in culture medium containing methotrexate (0.1 μ M). Transfectants expressing the fusion proteins are identified by ELISA analyses and are expanded in culture in order to generate fusion protein for

5 purification by binding to and elution from protein A Sepharose. Purified proteins in chromatography elution buffer are diafiltered into PBS and diluted to a final concentration of 100 μ g/ml. Balb/c mice are injected with 0.2 ml (20 μ g) of either the CD4-C γ 1 or CD4-C γ 4 fusion protein and the pharmacokinetics are tested as described in Example 1.3. The CD4-C γ 4 fusion protein has a significantly greater half-life than the

10 CD4-C γ 1 fusion protein.

Equivalents

The invention may be embodied in other specific forms without departing from the spirit or essential characteristics thereof. The foregoing embodiments are therefore to be considered in all respects illustrative rather than limiting on the invention described

15 herein. Scope of the invention is thus indicated by the appended claims rather than by the foregoing description, and all changes which come within the meaning and range of equivalency of the claims are intended to be embraced therein.

What is claimed is:

1. An antibody-based fusion protein with an enhanced circulating half-life, comprising at least a portion of an immunoglobulin (Ig) heavy chain having substantially reduced binding affinity for an Fc receptor, said portion of heavy chain being linked to a second non-Ig protein, said antibody-based fusion protein having a longer circulating half-life *in vivo* than an unlinked second non-Ig protein.
2. The antibody-based fusion protein of claim 1, wherein said portion of heavy chain comprises at least the CH2 domain of an ~~IgG2~~ or IgG4 constant region.
3. The antibody-based fusion protein of claim 1, wherein said portion of heavy chain comprises at least a portion of an IgG1 ~~constant~~ region having a mutation or a deletion at one or more amino acid selected from the group consisting of Leu₂₃₄, Leu₂₃₅, Gly₂₃₆, Gly₂₃₇, Asn₂₉₇, and Pro₃₃₁.
4. The antibody-based fusion protein of claim 1, wherein said portion of heavy chain comprises at least a portion of an IgG3 constant region having a mutation or a deletion at one or more amino acid selected from the group consisting of Leu₂₈₁, Leu₂₈₂, Gly₂₈₃, Gly₂₈₄, Asn₃₄₄, and Pro₃₇₈.
5. The antibody-based fusion protein of claim 1, wherein said portion of heavy chain further has binding affinity for an immunoglobulin protection receptor.
6. The antibody-based fusion protein of claim 1, wherein said portion of heavy chain has substantially reduced binding affinity for a Fc receptor selected from the group consisting of FcγRI, FcγRII and FcγRIII.
7. The antibody-based fusion protein of claim 1, wherein said second non-Ig protein is selected from the group consisting of a cytokine, a ligand-binding protein, and a protein toxin.

8. The antibody-based fusion protein of claim 1, wherein said cytokine is selected from the group consisting of a tumor necrosis factor, an interleukin, and a lymphokine.
9. The antibody-based fusion protein of claim 8, wherein said tumor necrosis factor is tumor necrosis factor alpha.
10. The antibody-based fusion protein of claim 8, wherein said interleukin is interleukin-2.
11. The antibody-based fusion protein of claim 8, wherein said lymphokine is a lymphotoxin or a colony stimulating factor.
12. The antibody-based fusion protein of claim 11, wherein said colony stimulating factor is a granulocyte-macrophage colony stimulating factor.
13. The antibody-based fusion protein of claim 1, wherein said ligand-binding protein is selected from the group consisting of CD4, CTLA-4, TNF receptor, and an interleukin receptor.
14. A method of increasing the circulating half-life of an antibody-based fusion protein, comprising the step of linking at least a portion of an Ig heavy chain to a second non-Ig protein, said portion of heavy chain having substantially reduced binding affinity for an Fc receptor, thereby forming an antibody-based fusion protein having a longer circulating half-life *in vivo* than an unlinked second non-Ig protein.
15. The method of claim 14, wherein said portion of heavy chain comprises at least the CH2 domain of an IgG2 or IgG4 constant region.
16. A method of increasing the circulating half-life of an antibody-based fusion protein, comprising the steps of:
 - (a) introducing a mutation or a deletion at one or more amino acid of an IgG1 constant region, said amino acid selected from the group consisting of Leu₂₃₄, Leu₂₃₅, Gly₂₃₆, Gly₂₃₇, Asn₂₉₇, and Pro₃₃₁, thereby producing an Ig

heavy chain having substantially reduced binding affinity for an Fc receptor; and

- (b) linking at least a portion of the heavy chain of step (a) to a second non-Ig protein,

thereby forming an antibody-based fusion protein having a longer circulating half-life *in vivo* than an unlinked second non-Ig protein.

17. A method of increasing the circulating half-life of an antibody-based fusion protein, comprising the steps of:
 - (a) introducing a mutation or a deletion at one or more amino acid of an IgG3 constant region, said amino acid selected from the group consisting of Leu₂₈₁, Leu₂₈₂, Gly₂₈₃, Gly₂₈₄, Asn₃₄₄, and Pro₃₇₈, thereby producing an Ig heavy chain having substantially reduced binding affinity for an Fc receptor; and
 - (b) linking at least a portion of the Ig heavy chain of step (a) to a second non-Ig protein,thereby forming an antibody-based fusion protein having a longer circulating half-life *in vivo* than an unlinked second non-Ig protein.
18. The method of claim 14, 16 or 17, wherein said portion of heavy chain further has binding affinity for an immunoglobulin protection receptor.
19. The method of claim 14, 16 or 17, wherein said portion of heavy chain has substantially reduced binding affinity for a Fc receptor selected from the group consisting of FcγRI, FcγRII and FcγRIII.
20. The method of claim 14, 16 or 17, wherein said second non-Ig protein is selected from the group consisting of a cytokine, a ligand-binding protein, and a protein toxin.
21. The method of claim 14, 16 or 17, wherein said cytokine is selected from the group consisting of a tumor necrosis factor, an interleukin, and a lymphokine.

22. The method of claim 21, wherein said tumor necrosis factor is tumor necrosis factor alpha.
23. The method of claim 21, wherein said interleukin is interleukin-2.
24. The method of claim 21, wherein said lymphokine is a lymphotoxin or a colony stimulating factor.
25. The antibody-based fusion protein of claim 24, wherein said colony stimulating factor is a granulocyte-macrophage colony stimulating factor.
26. The method of claim 14, 16 or 17, wherein said ligand-binding protein is selected from the group consisting of CD4, CTLA-4, TNF receptor, and an interleukin receptor.

Abstract of the Disclosure

Disclosed are methods for the genetic construction and expression of antibody-based fusion proteins with enhanced circulating half-lives. The fusion proteins of the present invention lack the ability to bind to immunoglobulin Fc receptors, either as a consequence of the antibody isotype used for fusion protein construction, or through directed mutagenesis of antibody isotypes that normally bind Fc receptors. The fusion proteins of the present invention may also contain a functional domain capable of binding an immunoglobulin protection receptor.

404PRHW4006/14.739141-1

1 XX GC1/118_HUMAN
1 XX GC3_HUMAN/118

41 XX GC1/118_HUMAN
41 XX GC3_HUMAN/118

81 XX A S T GC1/118_HUMAN
81 XX A S T GC3_HUMAN/118

121 KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS GC1/118_HUMAN
121 KGPSVFPLAP[C]S[R]STSGGTAALGCLVKDYFPEPVTVSWNS GC3_HUMAN/118

161 GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTYIC GC1/118_HUMAN
161 GALTSGVHTFPAVLQSSGLYSLSSVVTVPSSSLGTQTY[T]C GC3_HUMAN/118

201 NVNHKPSNTKVDKKVE----- GC1/118_HUMAN
201 NVNHKPSNTKVDK[R]VE[LKTPPLGDTTHTCPRCPPEPKSCDTP] GC3_HUMAN/118

217 ----- PKSCDKTHTCPPCPAPE GC1/118_HUMAN
241 PPCPRCPPEPKSCDTPPPCPRCPPEPKSCD[T]PPE[CP[R]C]PAPE GC3_HUMAN/118

234 LLGGPSVFLFPPKPKDTLMISRTPEVTCVVVDVSHEDPEV GC1/118_HUMAN
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274 KFNWYVDGVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDW GC1/118_HUMAN
321 [Q]F[X]WYVDGVEVHNAKTKPREEQYNST[F]RVVSVLTVLHQDW GC3_HUMAN/118

314 LNGKEYKCKVSNKALPAPIEKTISKAKGQPREPQVYTLPP GC1/118_HUMAN
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354 SRDELTKNQVSLTCLVKGFYPSDIAVEWESNGQPENNYKT GC1/118_HUMAN
401 SR[E]E[M]TKNQVSLTCLVKGFYPSDIAVEWES[S]GQPENNY[N]T GC3_HUMAN/118

394 TPPVLDSDGSFFLYSKLTVDKSRWQQGNVFSQSVMH EALH GC1/118_HUMAN
441 TPP[M]LDSDGSFFLYSKLTVDKSRWQQGN[T]FSQSVMH EALH GC3_HUMAN/118

434 NHYTQKSLSLSPGK GC1/118_HUMAN
481 N[R]E[T]QKSLSLSPGK GC3_HUMAN/118

FIG. 1

	10	20	30	40	
1	XX				GC1/118_HUMAN
1	XX				GC2/118_HUMAN
1	XX				GC4/118_HUMAN
	50	60	70	80	
41	XX				GC1/118_HUMAN
41	XX				GC2/118_HUMAN
41	XX				GC4/118_HUMAN
	90	100	110	120	
81	XX				GC1/118_HUMAN
81	XX				GC2/118_HUMAN
81	XX				GC4/118_HUMAN
	130	140	150	160	
121	KGPSVFPLAPSSKSTSGGTAALGCLVKDYFPEPVTVSWNS				GC1/118_HUMAN
121	KGPSVFPLAP[C]S[R]STSE[ST]AALGCLVKDYFPEPVTVSWNS				GC2/118_HUMAN
121	KGPSVFPLAP[C]S[R]STSE[ST]AALGCLVKDYFPEPVTVSWNS				GC4/118_HUMAN
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161	GALTSGVHTFPAVLQSSSGLYSLSSVVTVPSSSLGTQTYIC				GC1/118_HUMAN
161	GALTSGVHTFPAVLQSSSGLYSLSSVVTVPSSSLNFGTQTYT[C]				GC2/118_HUMAN
161	GALTSGVHTFPAVLQSSSGLYSLSSVVTVPSSSLGT[K]TY[T]C				GC4/118_HUMAN
	210	220	230	240	
201	NVNHKPSNTKVDK[K]VEPKSCDKTHTCPPCPAPELLGGPSV				GC1/118_HUMAN
201	NV[D]HKPSNTKVDK[T]VER[K][C]C---VECPPCPAP[P-V]A]GPSV				GC2/118_HUMAN
201	NV[D]HKPSNTKVDK[R]VE[S]K[Y]G---P]PCP[S]CPAPE[FL]GGPSV				GC4/118_HUMAN
	250	260	270	280	
241	FLFPPKPKD TLMISRTPEVTCVVVDVSHEDPEVKFNWYVD				GC1/118_HUMAN
237	FLFPPKPKD TLMISRTPEVTCVVVDVSHEDPEV[Q]FNWYVD				GC2/118_HUMAN
238	FLFPPKPKD TLMISRTPEVTCVVVDV[S]QEDPEV[Q]FNWYVD				GC4/118_HUMAN
	290	300	310	320	
281	GVEVHNAKTKPREEQYNSTYRVVSVLTVLHQDWLNGKEYK				GC1/118_HUMAN
277	GVEVHNAKTKPREEQ[F]NST[F]RVVSVLTV[V]H]HQDWLNGKEYK				GC2/118_HUMAN
278	GVEVHNAKTKPREEQ[F]NSTYRVVSVLTVLHQDWLNGKEYK				GC4/118_HUMAN
	330	340	350	360	
327	CKVSNKALPAPIEKTISKAKGQPREPQVYTLPPSRDELTK				GC1/118_HUMAN
317	CKVSNK[G]LPAPIEKTISK[T]KGQPREPQVYTLPPS[R]E[M]TK				GC2/118_HUMAN
318	CKVSNK[G]LP[S]S]IEKTISKAKGQPREPQVYTLPPS[Q]E[M]TK				GC4/118_HUMAN
	370	380	390	400	
361	NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD				GC1/118_HUMAN
357	NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPP[M]LD				GC2/118_HUMAN
358	NQVSLTCLVKGFYPSDIAVEWESNGQPENNYKTTPPVLD				GC4/118_HUMAN
	410	420	430	440	
401	DGSFFLYSKLTVDKSRWQQGNVFSQVMHEALHNHYTQKS				GC1/118_HUMAN
397	DGSFFLYSKLTVDKSRWQQGNVFSQVMHEALHNHYTQKS				GC2/118_HUMAN
398	DGSFFLYS[R]LTVDKSRWQ[E]GNVFSQVMHEALHNHYTQKS				GC4/118_HUMAN
441	LSLSPGK				GC1/118_HUMAN
437	LSLSPGK				GC2/118_HUMAN
438	LSLS[L]GK				GC4/118_HUMAN

FIG. 2

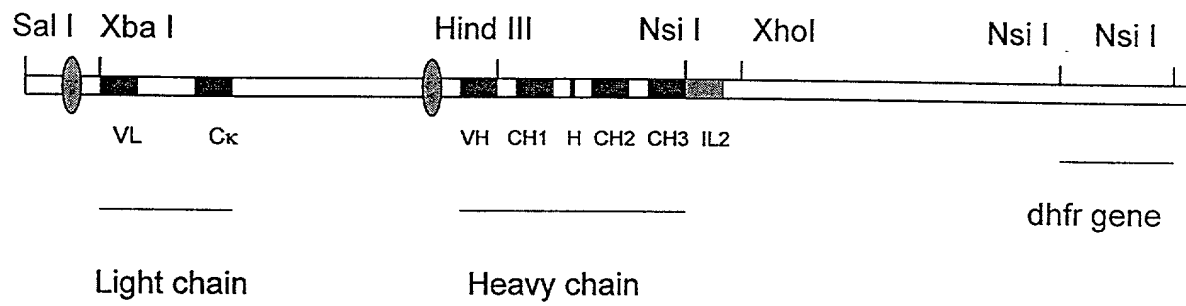


FIG. 3

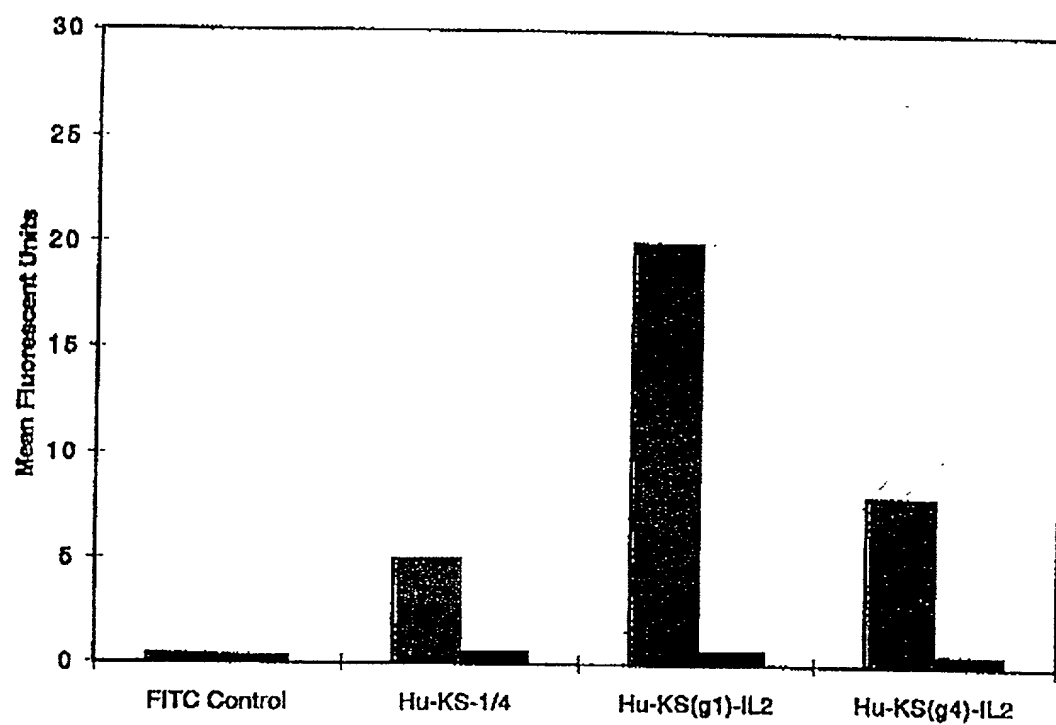


FIG. 4

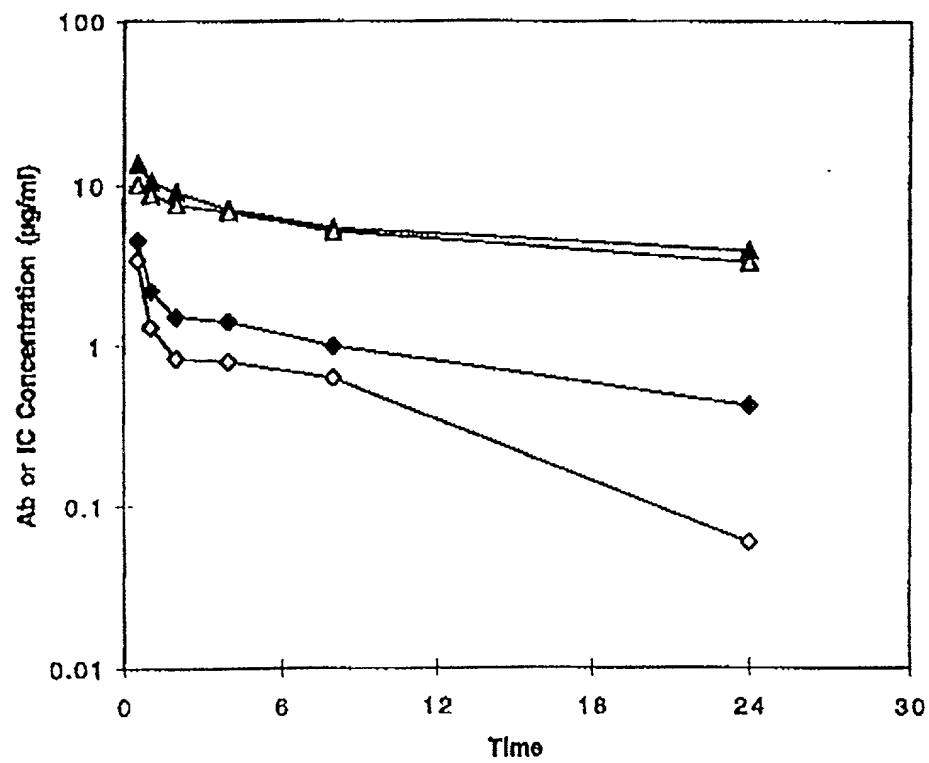
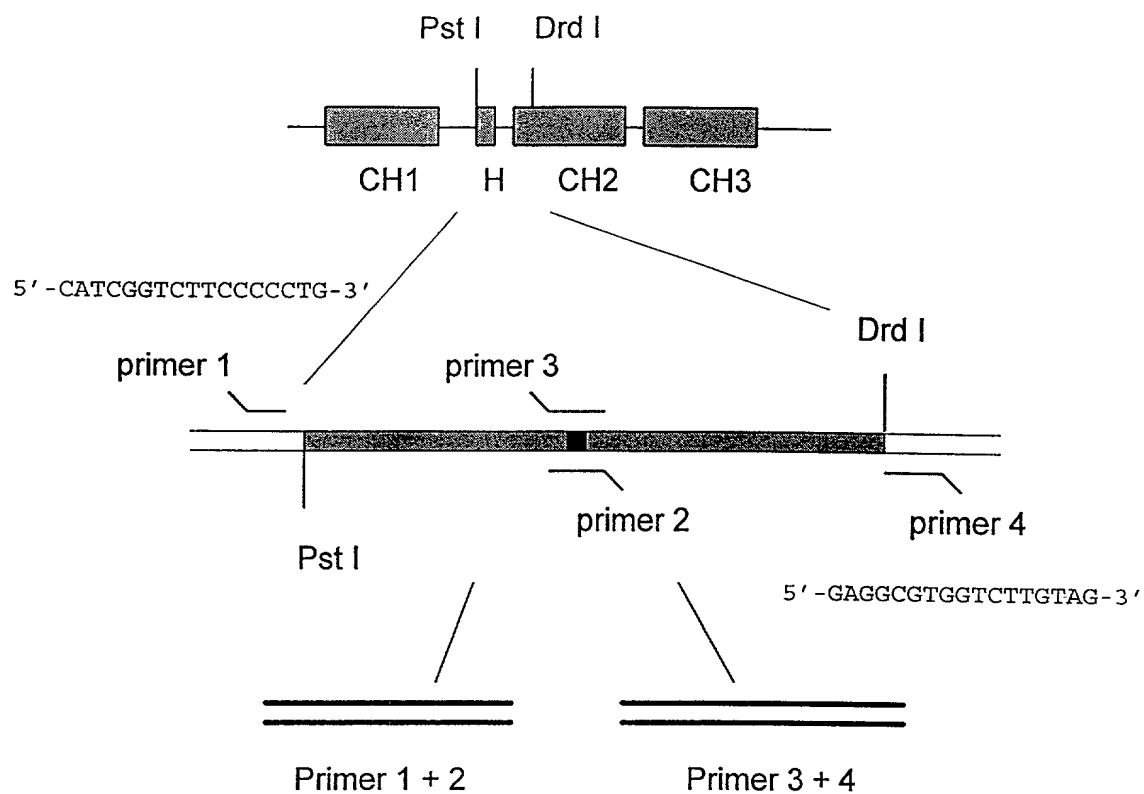
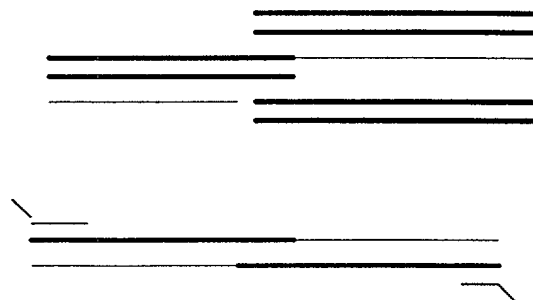


FIG. 5



Primer 3
 5' - TCTTCCTCAGCACCTCCCGTCGCAGGACCGTCAGTCTTCCTCTTC - 3'
 3' - GGTAGAGAAGGAGTCGTGGAGGGCAGCGTCCTGGC - 5'
 Primer 2

Mix PCR products, denature, anneal and extend



Amplify joined product with end primers 1 + 4

FIG. 6

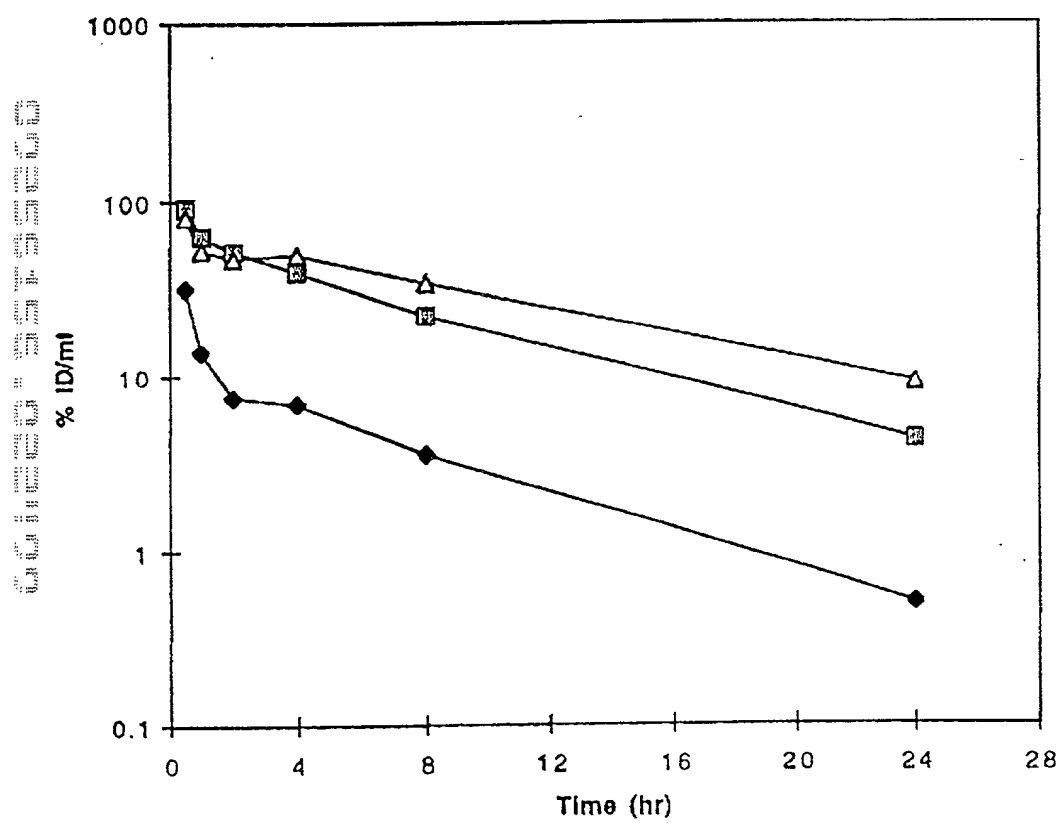


FIG. 7

SEQUENCE LISTING

<110> GILLIES, Stephen D
 LO, Kin-Ming
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<120> Enhancing the Circulating Half-life of
 Antibody-based Fusion Proteins

<130> LEX-003

<140>

<141>

<150> US 60/075,887

<151> 1998-02-25

<160> 8

<170> PatentIn Ver. 2.0

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Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
			20					25						30		
Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
		35					40						45			
Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
	50					55					60					
Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
65					70				75							80
Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
				85					90						95	
Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa	Xaa
			100					105						110		
Xaa	Xaa	Xaa	Xaa	Xaa	Ala	Ser	Thr	Lys	Gly	Pro	Ser	Val	Phe	Pro	Leu	
		115					120					125				
Ala	Pro	Ser	Ser	Lys	Ser	Thr	Ser	Gly	Gly	Thr	Ala	Ala	Leu	Gly	Cys	
	130					135					140					
Leu	Val	Lys	Asp	Tyr	Phe	Pro	Glu	Pro	Val	Thr	Val	Ser	Trp	Asn	Ser	
145					150					155					160	
Gly	Ala	Leu	Thr	Ser	Gly	Val	His	Thr	Phe	Pro	Ala	Val	Leu	Gln	Ser	
				165					170					175		

Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
 180 185 190
 Leu Gly Thr Gln Thr Tyr Ile Cys Asn Val Asn His Lys Pro Ser Asn
 195 200 205
 Thr Lys Val Asp Lys Lys Val Glu Pro Lys Ser Cys Asp Lys Thr His
 210 215 220
 Thr Cys Pro Pro Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val
 225 230 235 240
 Phe Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr
 245 250 255
 Pro Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu
 260 265 270
 Val Lys Phe Asn Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys
 275 280 285
 Thr Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Tyr Arg Val Val Ser
 290 295 300
 Val Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys
 305 310 315 320
 Cys Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile
 325 330 335
 Ser Lys Ala Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro
 340 345 350
 Pro Ser Arg Asp Glu Leu Thr Lys Asn Gln Val Ser Leu Thr Cys Leu
 355 360 365
 Val Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn
 370 375 380
 Gly Gln Pro Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser
 385 390 395 400
 Asp Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg
 405 410 415
 Trp Gln Gln Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu
 420 425 430
 His Asn His Tyr Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
 435 440 445

<210> 2

<211> 443

<212> PRT

<213> Homo sapiens

<220>

<223> IGG-2 CHAIN C REGION

<400> 2

Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa

1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17 18 19 20

1	5	10	15
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	20	25	30
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	35	40	45
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	50	55	60
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	65	70	75
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	85	90	95
Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa	100	105	110
Xaa Xaa Xaa Xaa Xaa Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu	115	120	125
Ala Pro Cys Ser Arg Ser Thr Ser Glu Ser Thr Ala Ala Leu Gly Cys	130	135	140
Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser	145	150	155
Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser	165	170	175
Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Asn	180	185	190
Phe Gly Thr Gln Thr Tyr Thr Cys Asn Val Asp His Lys Pro Ser Asn	195	200	205
Thr Lys Val Asp Lys Thr Val Glu Arg Lys Cys Cys Val Glu Cys Pro	210	215	220
Pro Cys Pro Ala Pro Pro Val Ala Gly Pro Ser Val Phe Leu Phe Pro	225	230	235
Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro Glu Val Thr	245	250	255
Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val Gln Phe Asn	260	265	270
Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr Lys Pro Arg	275	280	285
Glu Glu Gln Phe Asn Ser Thr Phe Arg Val Val Ser Val Leu Thr Val	290	295	300
Val His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val Ser	305	310	315
Asn Lys Gly Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser Lys Thr Lys	325	330	335
Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Arg Glu			

340 345 350
 Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly Phe
 355 360 365
 Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro Glu
 370 375 380
 Asn Asn Tyr Lys Thr Thr Pro Pro Met Leu Asp Ser Asp Gly Ser Phe
 385 390 395 400
 Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp Gln Gln Gly
 405 410 415
 Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His Tyr
 420 425 430
 Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
 435 440

<210> 3
 <211> 494
 <212> PRT
 <213> Homo sapiens

<220>
 <223> IGG-3 CHAIN C REGION

<400> 3
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 1 5 10 15
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 20 25 30
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 35 40 45
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 50 55 60
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 65 70 75 80
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 85 90 95
 Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa Xaa
 100 105 110
 Xaa Xaa Xaa Xaa Xaa Ala Ser Thr Lys Gly Pro Ser Val Phe Pro Leu
 115 120 125
 Ala Pro Cys Ser Arg Ser Thr Ser Gly Gly Thr Ala Ala Leu Gly Cys
 130 135 140
 Leu Val Lys Asp Tyr Phe Pro Glu Pro Val Thr Val Ser Trp Asn Ser
 145 150 155 160
 Gly Ala Leu Thr Ser Gly Val His Thr Phe Pro Ala Val Leu Gln Ser
 165 170 175

Ser Gly Leu Tyr Ser Leu Ser Ser Val Val Thr Val Pro Ser Ser Ser
 180 185 190
 Leu Gly Thr Gln Thr Tyr Thr Cys Asn Val Asn His Lys Pro Ser Asn
 195 200 205
 Thr Lys Val Asp Lys Arg Val Glu Leu Lys Thr Pro Leu Gly Asp Thr
 210 215 220
 Thr His Thr Cys Pro Arg Cys Pro Glu Pro Lys Ser Cys Asp Thr Pro
 225 230 235 240
 Pro Pro Cys Pro Arg Cys Pro Glu Pro Lys Ser Cys Asp Thr Pro Pro
 245 250 255
 Pro Cys Pro Arg Cys Pro Glu Pro Lys Ser Cys Asp Thr Pro Pro Pro
 260 265 270
 Cys Pro Arg Cys Pro Ala Pro Glu Leu Leu Gly Gly Pro Ser Val Phe
 275 280 285
 Leu Phe Pro Pro Lys Pro Lys Asp Thr Leu Met Ile Ser Arg Thr Pro
 290 295 300
 Glu Val Thr Cys Val Val Val Asp Val Ser His Glu Asp Pro Glu Val
 305 310 315 320
 Gln Phe Lys Trp Tyr Val Asp Gly Val Glu Val His Asn Ala Lys Thr
 325 330 335
 Lys Pro Arg Glu Glu Gln Tyr Asn Ser Thr Phe Arg Val Val Ser Val
 340 345 350
 Leu Thr Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys
 355 360 365
 Lys Val Ser Asn Lys Ala Leu Pro Ala Pro Ile Glu Lys Thr Ile Ser
 370 375 380
 Lys Thr Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro
 385 390 395 400
 Ser Arg Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val
 405 410 415
 Lys Gly Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Ser Gly
 420 425 430
 Gln Pro Glu Asn Asn Tyr Asn Thr Thr Pro Pro Met Leu Asp Ser Asp
 435 440 445
 Gly Ser Phe Phe Leu Tyr Ser Lys Leu Thr Val Asp Lys Ser Arg Trp
 450 455 460
 Gln Gln Gly Asn Ile Phe Ser Cys Ser Val Met His Glu Ala Leu His
 465 470 475 480
 Asn Arg Phe Thr Gln Lys Ser Leu Ser Leu Ser Pro Gly Lys
 485 490

<210> 4
 <211> 444

[illegible]

<212> PRT

<213> Homo sapiens

<220>

<223> IGG-4 CHAIN C REGION

<400> 4

Xaa 1	Xaa	Xaa	Xaa	Xaa 5	Xaa	Xaa	Xaa	Xaa	Xaa 10	Xaa	Xaa	Xaa	Xaa	Xaa 15	
Xaa	Xaa	Xaa	Xaa 20	Xaa	Xaa	Xaa	Xaa	Xaa 25	Xaa	Xaa	Xaa	Xaa	Xaa 30	Xaa	Xaa
Xaa	Xaa	Xaa 35	Xaa	Xaa	Xaa	Xaa	Xaa 40	Xaa	Xaa	Xaa	Xaa	Xaa 45	Xaa	Xaa	Xaa
Xaa	Xaa 50	Xaa	Xaa	Xaa	Xaa	Xaa 55	Xaa	Xaa	Xaa	Xaa	Xaa 60	Xaa	Xaa	Xaa	Xaa
Xaa 65	Xaa	Xaa	Xaa	Xaa	Xaa 70	Xaa	Xaa	Xaa	Xaa	Xaa 75	Xaa	Xaa	Xaa	Xaa	Xaa 80
Xaa	Xaa	Xaa	Xaa	Xaa 85	Xaa	Xaa	Xaa	Xaa	Xaa 90	Xaa	Xaa	Xaa	Xaa	Xaa 95	Xaa
Xaa	Xaa	Xaa	Xaa 100	Xaa	Xaa	Xaa	Xaa	Xaa 105	Xaa	Xaa	Xaa	Xaa	Xaa 110	Xaa	Xaa
Xaa	Xaa	Xaa 115	Xaa	Xaa	Ala	Ser	Thr 120	Lys	Gly	Pro	Ser	Val 125	Phe	Pro	Leu
Ala	Pro 130	Cys	Ser	Arg	Ser	Thr 135	Ser	Glu	Ser	Thr	Ala	Ala	Leu	Gly	Cys
Leu 145	Val	Lys	Asp	Tyr	Phe 150	Pro	Glu	Pro	Val	Thr 155	Val	Ser	Trp	Asn	Ser 160
Gly	Ala	Leu	Thr	Ser 165	Gly	Val	His	Thr	Phe 170	Pro	Ala	Val	Leu	Gln 175	Ser
Ser	Gly	Leu	Tyr 180	Ser	Leu	Ser	Ser	Val 185	Val	Thr	Val	Pro	Ser 190	Ser	Ser
Leu	Gly 195	Thr	Lys	Thr	Tyr	Thr	Cys 200	Asn	Val	Asp	His	Lys 205	Pro	Ser	Asn
Thr 210	Lys	Val	Asp	Lys	Arg	Val 215	Glu	Ser	Lys	Tyr	Gly 220	Pro	Pro	Cys	Pro
Ser 225	Cys	Pro	Ala	Pro	Glu 230	Phe	Leu	Gly	Gly	Pro 235	Ser	Val	Phe	Leu	Phe 240
Pro	Pro	Lys	Pro	Lys 245	Asp	Thr	Leu	Met	Ile 250	Ser	Arg	Thr	Pro	Glu 255	Val
Thr	Cys	Val	Val 260	Val	Asp	Val	Ser	Gln 265	Glu	Asp	Pro	Glu	Val 270	Gln	Phe
Asn	Trp	Tyr 275	Val	Asp	Gly	Val	Glu 280	Val	His	Asn	Ala	Lys 285	Thr	Lys	Pro
Arg 290	Glu	Glu	Gln	Phe	Asn	Ser 295	Thr	Tyr	Arg	Val	Val	Ser	Val	Leu	Thr

Val Leu His Gln Asp Trp Leu Asn Gly Lys Glu Tyr Lys Cys Lys Val
 305 310 315 320
 Ser Asn Lys Gly Leu Pro Ser Ser Ile Glu Lys Thr Ile Ser Lys Ala
 325 330 335
 Lys Gly Gln Pro Arg Glu Pro Gln Val Tyr Thr Leu Pro Pro Ser Gln
 340 345 350
 Glu Glu Met Thr Lys Asn Gln Val Ser Leu Thr Cys Leu Val Lys Gly
 355 360 365
 Phe Tyr Pro Ser Asp Ile Ala Val Glu Trp Glu Ser Asn Gly Gln Pro
 370 375 380
 Glu Asn Asn Tyr Lys Thr Thr Pro Pro Val Leu Asp Ser Asp Gly Ser
 385 390 395 400
 Phe Phe Leu Tyr Ser Arg Leu Thr Val Asp Lys Ser Arg Trp Gln Glu
 405 410 415
 Gly Asn Val Phe Ser Cys Ser Val Met His Glu Ala Leu His Asn His
 420 425 430
 Tyr Thr Gln Lys Ser Leu Ser Leu Ser Leu Gly Lys
 435 440

<210> 5
 <211> 17
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: primer 1

<400> 5
 catcgggtctt cccctg 17

<210> 6
 <211> 35
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: primer 2

<400> 6
 cggtcctgcg acgggaggtg ctgaggaaga gatgg 35

<210> 7
 <211> 45
 <212> DNA
 <213> Artificial Sequence

<220>
 <223> Description of Artificial Sequence: primer 3

<400> 7
 tcttctcag cacctcccgt cgcaggaccg tcagtcttcc tcttc 45

<210> 8

<211> 17
<212> DNA
<213> Artificial Sequence

<220>
<223> Description of Artificial Sequence: primer 4

<400> 8
gaggcgtggt cttgtag

17